PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/00, 7/08, 7/06, A61K 38/04, 39/00, G01N 33/53 — A1

(11) International Publication Number: **WO 95/18148**

(43) International Publication Date: 6 July 1995 (06.07.95)

(21) International Application Number: PCT/US93/11703

(22) International Filing Date: 28 December 1993 (28.12.93)

(71) Applicant: CHIRON MIMOTOPES PTY LTD [AU/AU]; 11 Duerdin Street, Clayton, VIC 3168 (AU).

(71)(72) Applicant and Inventor: GEYSEN, H., Mario [US/US]; 671 Brookview Avenue, Chapel Hill, NC 27594 (US).

(72) Inventor: RODDA, Stuart, J.; 11 Duerdin Street, Clayton, VIC 3168 (AU).

(74) Agents: GREEN, Grant, D. et al.; Chiron Corporation, Intellectual Property Dept.-R440, P.O. Box 8097, Emeryville, CA 94662-8097 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: T-CELL EPITOPES

(57) Abstract

T-cell epitopes are determined by assaying pools of peptides derived from the full length antigen sequence in the absence of xenogeneic serum, and determining the mitogenic effect of the peptides.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

T-Cell Epitopes

Description

Technical Field

This invention relates to the fields of molecular biology and immunology. More specifically, this invention relates to methods for determining T-cell epitopes and the specific peptide epitopes determined thereby.

Background of the Invention

T-cell determinants have for the most part been previously studied using clones of T-cells. By definition, the cells of such a clone are homogeneous and by their very nature are not representative of T-cells in the general population. Thus, generalization of discoveries made with these clones to the population as a whole is not possible.

A knowledge of the epitopes that stimulate T-cells in the general population would be invaluable in designing new diagnostic and therapeutic agents. For example, detection of an abnormally high proportion of T-cells to a particular antigen would allow earlier diagnosis of exposure to the antigen than standard tests requiring the production of specific antibodies. Similarly, detection of a population of T-cells sensitive to an antigen also would indicate a previous exposure, even when any antibody produced had become undetectable. Such methods would also be useful for monitoring the progress of vaccination. Knowledge of the epitopes that stimulate killer T-cells against particular antigens would create a new class of very specific anti-cancer agents. However, in order to use such agents, the T-cell determinants for each individual must be identified before treatment may commence.

One fundamental problem in determining T-cell epitopes in the general population is obtaining sufficient lymphocytes to carry out systematic studies. Furthermore, in every sample there will be an unknown and unknowable number of antigen-specific memory T-cells. In addition, there will be responses of some cell populations at a low frequency to components of the medium added to support cell growth in the assay. Thus, each test must be repeated a number of times to ensure that a reliable conclusion can be drawn. In many cases the supply of lymphocytes will be limited. For instance, in clinical conditions (for instance, infection with HIV) the patient may be able to provide samples containing only a small number of reactive T cells.

F. Sinigaglia *et al.*, Meth Enzymol (1991) 203:370-86 disclosed methods for determining T cell epitopes. D. Valmori *et al.*, J Immunol (1992) 149:717-21 disclosed T cell epitopes for tetanus toxin. A. Kumar *et al.*, J Immunol (1992) 148:1499-505 disclosed "universal" T cell epitopes to gp195 from *P. falciparum* (merozoite surface antigen).

Brief Description of the Figures

Fig. 1 depicts the sequence of HIV_{SF2} gp120 (31-509), used in Example 3.

Disclosure of the Invention

One aspect of the invention is a method for identifying T cell determinants, which method comprises reacting each of a plurality of pools with an immunological agent, the agent being immunologically reactive with the antigen, each pool comprising a plurality of overlapping peptide sequences of the antigen, assessing the strength of reaction between the agent and each of the pools, selecting one or more pools giving the strongest reaction with the agent, preparing a plurality of sub-pools, each sub-pool comprising one or a plurality of peptide sequences selected from one of the selected pools, reacting each of the sub-pools with the agent, and assessing the strength of reaction between the agent and each of the sub-pools. The agent is preferably a population or sample of peripheral blood mononuclear cells (PMBCs), where the strength of reaction is gauged by determining the amount of T cell activation that results.

Another aspect of the invention is a method for detecting the exposure of a subject to an antigen or pathogen by determining the response of a T cell-containing sample. Another aspect of the invention is a method for detecting the exposure of a subject to an antigen or pathogen by detecting binding of a T cell epitope peptide to a T cell antigen receptor.

Another aspect of the invention is an assay kit for detecting the exposure of a subject to an antigen or pathogen, comprising a T cell epitope, preferably in combination with means for detection of a mitogenic response or surface binding. Another aspect of the invention is an improved vaccine composition which comprises a T cell epitope peptide in combination with B cell epitope peptide.

Another aspect of the invention is a method for inducing immunity in a bird or mammal, by administering a composition comprising a T cell epitope and a B cell epitope specific for a pathogen.

Another aspect of the invention is a method to increase the number of T cells capable of responding to a pathogen *ex vivo*, by contacting T cells obtained from a subject with a T cell determinant, culturing the reactive T cells to increase the number of T cells capable of responding to the antigen, and administering said T cells to the subject.

Modes of Carrying Out The Invention

A. Definitions

The terms "T cell epitope" and "T cell determinant" refer to peptides or regions within a longer protein which bind T cell antigen receptors in conjunction with mammalian MHC proteins. Preferably, T cell epitopes are characteristic of a pathogen or malignancy. A "T cell epitope peptide" is a peptide of about 6 to about 20 amino acids, preferably about 8 to about 15 amino acids, which primarily consists of a T cell determinant. T cell epitopes are prepared from the primary sequence of antigens. A T cell epitope "having a sequence derived from" an antigen is a peptide which comprises a sequence of amino acids found consecutively within the antigen's primary sequence.

The terms "B cell epitope" and "B cell determinant" refer to antigens which are immunoreactive with an antibody or B cell surface antigen receptor (membrane-bound

antibody). B cell epitopes/determinants need not be proteins or peptides, but may include lipids, carbohydrates, and other molecules.

The term "vaccine" as used herein refers to a composition or formulation suitable for administration to a mammal or bird, and capable of inducing an immune response in the mammal or bird. Vaccines of the invention will include at least one T cell epitope and at least one B cell epitope, which need not be derived from the same antigen or pathogen. The B cell antigen may be presented as a whole protein or large fragment, or as whole killed pathogen, if desired. The T cell epitope is preferably included as a T cell epitope peptide.

The terms "label" and "detectable label" as used herein refer to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a protein or peptide. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ^{32}P and ^{125}I), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase is usually detected by its ability to convert 3,3',5,5'-tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may serve in several different modes. For example, ^{125}I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as an enzyme or as an antigen for a MAb. Further, one may combine various labels for desired effect. For example, MAbs and avidin also require labels in the practice of this invention: thus, one might label a peptide with biotin, and detect its presence with avidin labeled with ^{125}I, or with an anti-biotin MAb labeled with HRP. Other permutations and possibilities will be readily apparent to those of ordinary skill in the art, and are considered as equivalents within the scope of the instant invention.

The term "biological sample" refers to any sample obtained from a bird or mammal which contains live T cells, preferably peripheral blood mononuclear cells. Suitable biological samples are typically derived from blood, but may be derived from any biological

tissue or fluid (*e.g.*, biopsy specimens, lymph, pus, saliva, semen, and the like) where T cells may be found.

A "kit" within the scope of this invention includes at least one T cell epitope peptide, and printed instructions for performing an assay. "Printed instructions" may be written or printed on paper or other media, or committed to electronic media such as magnetic tape, computer-readable disks or tape, CD-ROM, and the like. Kits also preferably include means for detecting positive responses, for example labels for the T cell epitope peptides, ^{3}H-T, or similar means. Kits may also include culture dishes, culture reagents, and other such supporting materials.

The term "pharmaceutically acceptable carrier" means refers to compounds and compositions which may be administered to mammals and/or birds without undue toxicity. Exemplary pharmaceutically acceptable salts include mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co.).

The term "effective amount" refers to the amount of T cell epitope peptide and B cell antigen required to effect an immune response in a subject. The precise effective amount will vary from subject to subject, depending on age, species, size, weight, and general health, but will generally correspond to the amount effective for traditional vaccines.

B. General Method

This invention involves the combination of various test compounds in a plurality of pools, and testing the pools for immunoreactivity with T cells. The pools are tested for activity, and selected active pools resynthesized as a plurality of sub-pools, generally derived from the sequences of the first pool. The plurality of sub-pools is then assayed, and active sub-pools selected for resynthesis as a plurality of sub-sub-pools. This process is reiterated as many times as desired, preferably until the sub-pools contain only a single species of peptide, thus identifying the active peptides. It is essential to minimize the number of assays performed, as one must use peripheral blood lymphocytes (PBLs) to obtain an accurate

reading. This process minimizes the number of assays which must be performed, thus making possible T cell epitope determination for an individual.

The size of the pool will depend on the number of active test compounds expected to be discovered. Obviously, the major benefit of the invention will be realized where the number of active test compounds is a small proportion of the compounds to be screened. To minimize the amount of test material required for the assays, the number of test compounds in each pool should be chosen so that the majority of pools will be inactive.

T cell epitopes are primarily or solely composed of peptides. Protein antigens are digested by antigen-presenting cells (APCs), and fragments of the antigens presented on the APC surface in the context of a major histocompatibility complex (MHC) protein. Thus, T cell epitopes are always linear fragments of the native antigen: there are no discontinuous epitopes, as in the case of B cell epitopes. Accordingly, T cell epitopes may be determined by preparing a series of peptides which span the amino acid sequence of the antigen. The peptides are selected to overlap, so that determinants are not missed due to straddling the junction between two test peptides. In general, one should allow at least 12-13 residues for a T cell determinant. Thus, if the peptides used are 13mers, one should prepare overlapping 13mers that overlap by 12 residues (*i.e.*, that are offset by one residue, *e.g.*, $^{1}XXXXXXXXXXXXX^{13}$, $^{2}XXXXXXXXXXXXX^{14}$, *etc.*). If longer peptides are used, the offset may be increased accordingly. Thus, if one employs 15mers, one may synthesize a series of peptides in which each is offset from the next by two or three residues (*e.g.*, $^{1}XXXXXXXXXXXXXXX^{15}$, $^{3}XXXXXXXXXXXXXXX^{17}$, *etc.*).

The peptides may be synthesized by any convenient method. A presently preferred method is the synthetic scheme disclosed in WO90/09395, in which peptides are synthesized bound to a plurality of plastic pins via a cleavable linkage. Following synthesis, the peptides are cleaved from the support and are combined into pools for testing. For ease of analysis, the peptides are preferably grouped into pools on the basis of their sequences, *i.e.*, the first ten sequential peptides in pool 1, the next ten peptides in pool 2, *etc.* However, other grouping strategies may also be useful. For example, one may wish to group peptides derived from similar sections of a protein having internal repeats (*e.g.*, pool 1 may contain the first several peptides from the beginning of each immunoglobulin fold in an antibody).

T cells are obtained from a subject, and are contacted with the peptide pools. For determination of T cell epitopes specific to a given pathogen, one should select subjects who have been exposed to the pathogen, either as the result of infection or vaccination. Subjects who have recovered from infection may provide the best (most protective/diagnostic) epitopes. The T cells may be either fresh or frozen, but are not cultured or cloned prior to assay in order to preserve the natural distribution of T cells having different antigen receptors. The T cells are preferably obtained from peripheral blood as PBMCs, and are separated from erythrocytes and polymorphonuclear cells by centrifugation or fluorescence-activated cell sorting. The T cells and peptide pools are placed together in a plurality of wells or culture dishes, and the response determined by determining binding directly (*e.g.*, by fluorescence of a labeled peptide) or through mitogenicity (typically measured by ^{3}H-T uptake after culture). The cells are preferably cultured in autologous serum, in the absence of xenogeneic or pooled serum, to reduce background responses. T cells which recognize one or more of the peptides respond by mitosis, clonally expanding the number of specific T cells recognizing the peptide determinant. One may optionally add autologous APCs, either as live cells or as fixed cells, or one may culture the T cells in wells having an autologous MHC protein bound to a support, for improved antigen presentation.

Once T cell epitopes have been determined, they may be employed as reagents in T cell-based immunoassays. Such assays are advantageous over antibody-based assays because they do not require that the subject have already mounted a detectable antibody response to the antigen. The T cell (T_H) response to an antigen necessarily precedes the B cell (antibody) response: thus, the T cell response may be detected earlier than the antibody response. Diagnostic assays may be performed with a much smaller sample, because it is not necessary to scan the entire length of the antigen once the epitopes have been determined. It is likely that some antigens will exhibit different epitopes in different individuals, based on the heterogeneity of the MHC proteins which present the antigen to the immune system. However, one may include a number of T cell epitope peptides in each assay. For example, one may screen a subject's PBMCs against a pool comprising all known HIV_{SF2} gp120 T cell epitopes. Response to any peptide in the pool may be counted as a positive response.

The response may be detected by a variety of methods. The presently preferred method is to culture PBMCs in contact with the peptide pools, followed by pulsing with ^{3}H-T to determine T cell proliferation. One may also detect mitogenic effects by other means, for example, by monitoring the increase in interleukin-2 mRNA using PCR, and the like. Alternatively, one may label the peptides and detect binding directly to the T cell antigen receptor. For example, the peptides may be labeled with fluorescein.

Immune responses in birds and mammals are believed to require interaction between T cells and B cells, where both cell types are activated by contact with an appropriate antigen. Accordingly, effective vaccines should include both B cell and T cell epitopes. Suitable vaccine formulations are known in the art for use with the epitopes of the invention. See for example, EP 399 843, which discloses an adjuvant emulsion formulation.

C. Examples

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

Example 1

(*M. bovis* Determinants)

A) In this assay, the stimulation of T cells is detected by their proliferation after exposure to the test compounds. It will be appreciated that other assay methods can be used without violating the essence of the invention.

Autologous serum was obtained after defibrination of 50 to 100 mL freshly-drawn human venous blood by gentle agitation with 3-5 g of sterile acid-washed glass balls (5-8 mm diameter) for at least 10 min. Serum was collected from above PBMC bands after density interface centrifugation. Autologous plasma was collected from above the PBMC band after density interface centrifugation of anticoagulated whole blood. Group AB human plasmas, anticoagulated with either acid-citrate-dextrose or citrate-phosphate-dextrose (ACD, CPD), were a gift from Commonwealth Serum Laboratories (CSL, Melbourne, Australia). Plasma was converted into serum by the addition of 10 mM $CaCl_2$ and 1 IU/mL (final) of human thrombin (CSL) to plasma prewarmed to 37°C, followed by vigorous agitation for 5-10 min.

The mixture was allowed to stand at room temp. for 60 min and the supernatant serum was collected by centrifugation at an RCF of 20000 at 4°C for 20 min. Sera or plasmas were heat-inactivated at 56°C for 45 min in a water bath.

PBMC were obtained from heparinized or defibrinated whole blood from a healthy adult volunteer donor panel. Alternatively, screened buffy coats from blood anticoagulated with CPD, kindly supplied by the Red Cross Blood Bank (Melbourne, Australia) were used. Blood was diluted slightly to between 1:1 and 2.5:1 (50% to 72% (v/v) whole blood) with incomplete medium and underlayed with Ficoll/Paque (Pharmacia LKB, Uppsala, Sweden) in a 50 mL polypropylene centrifuge tube in a final ratio of 2:1 diluted blood:Ficoll/Paque. A band of PBMC was isolated at the interface by centrifugation at an RCF of 450 for 25 min. When autologous serum was required, it was recovered from above the PBMC band. PBMC from the band were washed twice by centrifugation in "incomplete" medium containing about 10% (v/v) added autologous serum. The first wash was at an RCF of 450 for 15 min to ensure efficient pelleting of cells from the medium containing residual Ficoll/Paque, while the second was at an RCF of 150 for 10 min to minimize platelet and debris contamination. Cells were suspended in complete medium containing autologous serum or pooled human serum and viable cells were counted on a hemocytometer using trypan blue dye exclusion.

All overlapping 12-mer peptides of the protein MPB 70, produced by the bacterium *Mycobacterium bovis*, were synthesized. This protein is one that is known to stimulate T cells, and has the following primary sequence:

Gly Asp Leu Val Gly Pro Gly Cys Ala Glu
Tyr Ala Ala Ala Asn Pro Thr Gly Pro Ala
Ser Val Gln Gly Met Ser Gln Asp Pro Val
Ala Val Ala Ala Ser Asn Asn Pro Glu Leu
Thr Thr Leu Thr Ala Ala Leu Ser Gly Gln
Leu Asn Pro Gln Val Asn Leu Val Asp Thr
Leu Asn Ser Gly Gln Tyr Thr Val Phe Ala
Arg Thr Asn Ala Ala Phe Ser Lys Leu Pro
Ala Ser Thr Ile Asp Glu Leu Lys Thr Asn
Ser Ser Leu Leu Thr Ser Ile Leu Thr Tyr
His Val Val Ala Gly Gln Thr Ser Pro Ala
Asn Val Val Gly Thr Arg Gln Thr Leu Gln
Gly Ala Ser Val Thr Val Thr Gly Gln Gly
Asn Ser Leu Lys Val Gly Asn Ala Asp Val
Val Cys Gly Gly Val Ser Thr Ala Asn Ala

Thr Val Tyr Met Ile Asp Ser Val Leu Met
Pro Pro Ala

The 152 peptides were synthesized in quadruplicate on plastic rods according to the methods disclosed in WO90/09395. The base-labile moiety Lys-Pro was incorporated at the carboxy terminal of each peptide, and the amino terminal amine group was acetylated. The peptides were cleaved from the rods into 150 μL of 0.1 M bicarbonate buffer at pH 8.2, and the solutions of each different peptide were combined into pools.

Fourteen pools of peptides were prepared. Each pool consisted of 11 adjacent overlapping 12-mer peptides. Thus, Pool #1 consisted of the 11 peptides ^{1}GDLVGPGGCA-EYA to ^{11}YAAANPTGPASV (where the superscript indicates the residue within the MPB 70 sequence above). Pools were made by mixing 200 μL volumes of each of the peptide preparations. To each peptide pool was added 1.8 mL of RPMI-1640 cell culture medium. Each assay was carried out in 48 replicates. Lymphocytes were separated from whole blood (50 mL) and resuspended in RPMI complete medium at a cell concentration of approximately 1.07×10^6 cells/mL. In the assay, 180 μL cell suspension was added to 20 μL of peptide pool in microculture plates and incubated for 7 days at 37°C in a 5% CO_2 atmosphere. Six hours before harvest, the cultures were pulsed with 1 μCi of ^{3}H-T. The cells were harvested, and the amount of ^{3}H-T incorporated measured in a liquid scintillation counter (LKB model 1205 Betaplate). The positive control was 20 μL of a 100 μg/mL solution of MPB-70, and the negative control was 20 μL of cell culture medium.

A threshold point was calculated based on the probability that no assay result would exceed the threshold by chance (on a two-tailed test). This procedure was repeated until all assay results below the threshold were included in the "background" estimate and change was made in the estimate of the threshold. Thus, when the data were submitted to this procedure, the lowest 615 assay results (of 768 total) yielded a mean of 3170 cpm (standard deviation 1420) and gave a threshold estimate of 7700 cpm. Thus, from this assay, we would not expect any assay result to return a value greater than 7700 cpm if there was no specific proliferation of the lymphocytes. In this assay, 153 results returned values greater than this. Pools 1 and 2 returned 33 and 40 positive results (out of 48 assays), respectively, as shown in Table 1:

TABLE 1: Positive Results per Pool

Pool	Number of Positives	Mean cells per assay	Frequency (1 in...)
1	33	1.163	172,000
2	40	1.792	111,600
3	4	0.087	2,298,900
4	5	0.110	1,818,200
5	3	0.065	3,076,900
6	0	<0.021	>9,523,800
7	3	0.065	3,076,900
8	3	0.065	3,076,900
9	2	0.043	4,651,200
10	1	0.021	9,523,800
11	0	<0.021	>9,523,800
12	2	0.043	4,651,200
13	3	0.065	3,076,900
14	6	0.134	1,492,500
Control (+)	48	>3.871	<52,700
Control (-)	0	<0.021	>9,523,800

The analysis indicates that only pools 1 and 2 contain peptides which significantly stimulate the growth of T cells in the blood obtained from this particular donor.

B) The assay was repeated with 20 μL of the individual peptide preparations that were pooled to make pools 1 and 2. In this case each assay was carried out with 12 replicates. The results are shown in Table 2:

TABLE 2: Assay of Pools 1 and 2

Peptide	Number of Positives	Mean cells per assay	Frequency (1 in...)
1 GDLVGPGCAEYA	0	<0.087	>2,300,000
2 DLVGPGCAEYAA	0	<0.087	>2,300,000
3 LVGPGCAEYAAA	0	<0.087	>2,300,000
4 VGPGCAEYAAAN	1	0.087	2,300,000
5 GPGCAEYAAANP	0	<0.087	>2,300,000
6 PGCAEYAAANPT	0	<0.087	>2,300,000
7 GCAEYAAANPTG	1	0.087	2,300,000
8 CAEYAAANPTGP	1	0.087	2,300,000
9 AEYAAANPTGPA	4	0.405	493,800
10 EYAAANPTGPAS	5	0.539	371,100
11 YAAANPTGPASV	4	0.405	493,800
12 AAANPTGPASVQ	4	0.405	493,800
13 AANPTGPASVQG	3	0.288	694,400
14 ANPTGPASVQGM	6	0.693	288,600
15 NPTGPASVQGMS	4	0.405	493,800
16 PTGPASVQGMSQ	1	0.087	2,300,000
17 TGPASVQGMSQD	2	0.182	1,098,900
18 GPASVQGMSQDP	0	<0.087	>2,300,000
19 PASVQGMSQDPV	0	<0.087	>2,300,000
20 ASVQGMSQDPVA	0	<0.087	>2,300,000
21 SVQGMSQDPVAV	1	0.087	2,300,000
22 VQGMSQDPVAVA	0	<0.087	>2,300,000
Positive Control	12	>2.485	<80,000
Negative Control	0	<0.087	>2,300,000

The results suggest that there are two peptides that have a major stimulatory effect on the T cells. These are peptide 14 (ANPTGPASVQGM) and peptide 10 (EYAAANPTGPAS), although other peptides that share parts of the sequences of these peptides do have a partial stimulatory effect.

Example 2

(Herpes Simplex Virus Determinants)

Peripheral blood mononuclear cells (PBMCs) were collected from ten donors as described below, and frozen until use.

Sample Collection/Preparation:

PBMCs are prepared and preserved from 45 mL of whole blood. The cells should not be exposed to bovine or other xerosera. Replicate numbers (>24) are necessary to assure reasonable confidence in the value calculated. The number of cells seeded per well depends on the expected frequency of responding T cells in the preparation.

Fresh heparinized whole blood (45 mL) is partitioned in three 50 mL conical centrifuge tubes, then diluted with 10 mL of PBS warmed to room temperature. The diluted blood is carefully underlaid with 10 mL Ficoll, taking care not to mix the interface, then centrifuged in a swinging bucket rotor at 400 × g for 20 min at 20°C with the brake off, between 18-22°C. The serum is aspirated from above the lymphocyte band and the cells removed from the interface, taking care to avoid removing material from the Ficoll layer (which contains granulocytes). Each band should be collected in about 5 mL. The PBMC from the three tubes are combined into a 50 mL centrifuge tube and diluted with 4 volumes of PBS, then centrifuged at 60-100 × g for 8-10 min at 20°C with the brake on. The supernatants are decanted, and the cell pellets resuspended in 10 mL PBS. The cells are transferred to a 15 mL tube and centrifuge as before. The PBMC are washed one more time with 10 mL PBS. Cells are resuspended in 10 mL RPMI CM-1% PHS and counted in a hemacytometer using trypan blue exclusion to estimate viability. If a significant red cell contamination is evident, dilute the PBMC to be counted in Turk's solution. The cells are pelleted again, and resuspended for freezing in 1.0 mL of RPMI CM containing 20% PHS. RPMI CM (1.0 mL) containing 20% PHS and 20% DMSO is added dropwise while swirling the tube, and the cells transferred to two Nunc freezing vials. The vials are placed on ice until all are filled, then frozen at -70°C until used.

Assay plates are prepared before thawing the PBMC. Use 96-well U-bottom plates for cultures seeded with >20,000 cells/well; 96-well V-bottom plates for cultures seeded with <20,000 cells/well. Antigens are diluted in lymphocyte basal medium to 10× the final concentration, and are added in 20 μL to the wells. The PHA is added 48 hours before the assay is completed. Basal medium alone is added to the "cells alone" wells. Vials of frozen PBMC are retrieved from the liquid N_2 and kept on dry ice until they ready to be thawed. Six to eight samples can be processed at a time. Conical centrifuge tubes (15 mL) and an

equal number of 60 mm tissue culture dishes labeled with the sample ID are set up. Lymphocyte wash medium (5 mL) is added to each of the tubes and dishes, and vials corresponding to the samples to be processed placed in a 37°C water bath. The vials are removed from the water bath before each sample is completely thawed, the outside rinsed with ethanol, and the contents placed in it's respective dish. One mL of medium from the 15 mL tube is used to rinse the vial. The cell suspension is transferred from the thawing dish to the centrifuge tube, and the dish rinsed with the medium used to rinse the vial. The PBMC are pelleted in a tabletop centrifuge at 900 × g for 8 min, and the pellets washed twice with 10 mL of wash medium, then resuspended in 5 mL of lymphocyte culture medium. The cells are counted after the second wash and the volume of medium and cells needed to set up the plate calculated. To seed 96 wells with 180 μL of cell suspension/well, 18.5 mL of cell suspension is needed. The number of cells seeded per well depends on the expected responder frequency. If the expected frequency is low (*e.g.* 1 responder in 500,000 PBMC), 10^5 cells/well is a good starting point. For expected frequencies of 1 in 20,000 to 1 in 10,000 about 20,000 cells/well should be used. The object is to achieve roughly half of the antigen-containing wells scored as positive. The washed cells are diluted such that the number of cells/well will be delivered in 180 μL to the U-bottom plates. When V-bottom plates are used for high-frequency assays, the cells are resuspended at 1-3 × 10^5 cells/mL and the appropriate volume added. As little as 20 μL of cells can be seeded in V-bottom wells. However, in this configuration, the cells should be diluted in medium containing antigen rather than adding the antigen to the wells prior to thawing the PBMC.

The plates are incubated in a humidified 7% CO_2 incubator. Two days before the pulse, PHA is added to the appropriate wells in 20 μL of basal medium. Early on day 6, 20 μL of ^{3}H-thymidine diluted to 25 mCi/mL in basal medium is added to each well using a multichannel pipettor, and the plates returned to the incubator. After six hours, the plates are harvested with a Cambridge 2800 harvester using program 0. Alternatively, the plates can be frozen and harvested at a later date. The filter mats are dried for several hours or overnight before sealing them into their bags with 10 mL of scintillation fluid. The filters are counted in the β-plate counter using the appropriate protocol.

Antigen Preparation:

Peptides 13mers were prepared in quadruplicate on plastic rods according to the methods disclosed in WO99/09395, as described in the Example above, based on the amino acid sequence of the Herpes simplex virus 2 antigen gD2:

Lys Tyr Ala Leu Ala Asp Pro Ser Leu Lys
Met Ala Asp Pro Asn Arg Phe Arg Gly Lys
Asn Leu Pro Val Leu Asp Gln Leu Thr Asp
Pro Pro Gly Val Lys Arg Val Tyr His Ile
Gln Pro Ser Leu Glu Asp Pro Phe Gln Pro
Pro Ser Ile Pro Ile Thr Val Tyr Tyr Ala
Val Leu Glu Arg Ala Cys Arg Ser Val Leu
Leu His Ala Pro Ser Glu Ala Pro Gln Ile
Val Arg Gly Ala Ser Asp Glu Ala Arg Lys
His Thr Tyr Asn Leu Thr Ile Ala Trp Tyr
Arg Met Gly Asp Asn Cys Ala Ile Pro Ile
Thr Val Met Glu Tyr Thr Glu Cys Pro Tyr
Asn Lys Ser Leu Gly Val Cys Pro Ile Arg
Thr Gln Pro Arg Trp Ser Tyr Tyr Asp Ser
Phe Ser Ala Val Ser Glu Asp Asn Leu Gly
Phe Leu Met His Ala Pro Ala Phe Glu Thr
Ala Gly Thr Tyr Leu Arg Leu Val Lys Ile
Asn Asp Trp Thr Glu Ile Thr Gln Phe Ile
Leu Glu His Arg Ala Arg Ala Ser Cys Lys
Tyr Ala Leu Pro Leu Arg Ile Pro Pro Ala
Ala Cys Leu Thr Ser Lys Ala Tyr Gln Gln
Gly Val Thr Val Asp Ser Ile Gly Met Leu
Pro Arg Phe Ile Pro Glu Asn Gln Arg Thr
Val Ala Leu Tyr Ser Leu Lys Ile Ala Gly
Trp His Gly Pro Lys Pro Pro Tyr Thr Ser
Thr Leu Leu Pro Pro Glu Leu Ser Asp Thr
Thr Asn Ala Thr Gln Pro Glu Leu Val Pro
Glu Asp Pro Glu Asp Ser Ala Leu Leu Glu
Asp Pro Ala Gly Thr Val Ser Ser Gln Ile
Pro Pro Asn Trp His Ile Pro Ser Ile Gln
Asp Leu Ala Pro His His Ala Pro Ala Ala
Pro Ser Asn Pro Gly Leu Ile Ile Gly Ala
Leu Ala Gly Ser Thr Leu Ala Ala Leu Val
Ile Gly Gly Ile Ala Phe Trp Val Arg Arg
Arg Ala Gln Met Ala Pro Lys Arg Leu Arg
Leu Pro His Ile Arg Asp Asp Asp Ala Pro
Pro Ser His Gln Pro Leu Phe Tyr

Twelve pools of peptides were prepared. Pool 1 contained 12 peptides, pools 2-11 contained 10 peptides, and pool 12 contained 8 peptides. Pools were made by mixing 200 μL volumes of each of the peptide preparations. To each peptide pool was added 1.8 mL of RPMI-1640 cell culture medium. Each assay was carried out in 48 replicates. In the assay, 180 μL cell suspension was added to 20 μL of peptide pool in microculture plates.

Results:

Each pool was scored for strength of response (none, weak, moderate, high) for each donor. Pools 1-3 and 7-12 exhibited weak to zero response. Pools 4-6 exhibited at least moderate responses for 7, 6, and 5 of the donors, respectively.

For three of the donors, samples were retested against individual peptides from the responding pools to determine which peptides were responsible for the observed activity.

Donor A responded to peptides in the following regions: ^{19}GKNLPVLDQL, ^{97}IAWYRMGDNCAIPITV, ^{161}AGTYLRLV, and ^{202}LTSKAYQQG. Donor C responded to peptides in the following regions: ^{136}CPIRTQPRWSYYDSF, and ^{170}INDWTEITQFILE. Donor D responded to peptides in the following regions: ^{133}PRWPRWSYYDSFS-AVSEDNLGFLMHAPAFETAGTYLRLVKINDWTEITQFILEHRARASCKYAL (not resolved), and ^{232}ALYSLKIAGWHGPKP. Samples from Donor D were additionally tested for CD8+ T cell epitopes. The following CD8 epitope was determined: ^{307}APAAPSNPG. Interestingly, the CD8 epitope occurs in a region which is different in HSV-1 and HSV-2, and may account for the fact that immune responses to HSV are type-specific.

Example 3

(Human Immunodeficiency Virus Determinants)

Eight subjects were selected from participants in a clinical study to examine a recombinant HIV vaccine based on HIV_{SF2} gp120. The subjects were vaccinated with HIV_{SF2} gp120 in an adjuvant formulation on day 0, and boosted at one month and six months. Some subjects received placebos. Samples were obtained and treated as described in Example 2 above, both prior to vaccination and two weeks following the last boost.

Peptides were prepared as described in Example 2 above. The peptides were 15mers, offset by two residues, spanning the length of HIV_{SF2} gp120 (excluding the 30 residue signal

sequence). The gp120 sequence is shown in Figure 1. The peptides were grouped in pools of 13 peptides, for a total of 18 pools. The results are shown in Table 3.

TABLE 3: Assay of HIV Pools

Pool	Peptide sequence	Negative Ab pre	Negative Ab post	Positive Ab pre	Positive Ab post
1	31-69	0/2	0/2	2/6	1/6
2	57-95	0/2	0/2	0/6	0/6
3	**83-121**	**0/2**	**0/2**	**0/6**	**6/6**
4	109-147	0/2	1/2	1/6	0/6
5	135-173	1/2	0/2	0/6	0/6
6	161-199	0/2	0/2	2/6	0/6
7	187-225	2/2	1/2	6/6	6/6
8	213-251	1/2	0/2	2/6	2/6
9	239-277	1/2	1/2	0/6	0/6
10	265-303	2/2	2/2	5/6	0/6
11	**291-329**	**1/2**	**1/2**	**1/6**	**6/6**
12	317-355	0/2	1/2	3/6	3/6
13	343-381	0/2	0/2	1/6	3/6
14	369-407	1/2	1/2	1/6	0/6
15	395-433	0/2	0/2	1/6	0/6
16	421-459	0/2	0/2	0/6	1/6
17	447-485	0/2	0/2	0/6	0/6
18	473-509	0/2	0/2	1/6	3/6
gp120	31-509	0/2	0/2	0/6	6/6

In Table 3, "Negative Ab" indicates the two subjects who did not produce antibodies to gp120 detectable by ELISA (and thus were probably placebo recipients), while "Positive Ab" indicates the six subjects who did produce gp120 antibodies. Full length (31-509) gp120 was included as a positive control.

The results indicated that pools 3 and 11 probably contain the T cell determinants for HIV_{SF2} gp120, based on the increase in response between pre-vaccination and post vaccination tests. Pools 3 (peptides 27-39) and 11 (peptides 121-133) were analyzed for five subjects to determine which peptides were responsible for the observed activity. Pools for

which responses were obtained both before and after vaccination probably indicate cross-reactive with other pathogens. The results are shown in Table 4.

TABLE 4: Assay of Pools 3 and 11

Peptide	Sequence	Response post-vaccination
27	^{83}VVLGNVTENFNMWKN	0/5
28	^{85}LGNVTENFNMWKNNM	1/5
29	^{87}NVTENFNMWKNNMVE	2/5
30	^{89}TENFNMWKNNMVEQM	5/5
31	^{91}NFNMWKNNMVEQMQE	5/5
32	^{93}NMWKNNMVEQMQEDI	5/5
33	^{95}WKNNMVEQMQEDIIS	3/5
34	^{97}NNMVEQMQEDIISLW	1/5
35	^{99}MVEQMQEDIISLWDQ	0/5
36	^{101}EQMQEDIISLWDQSL	1/5
37	^{103}MQEDIISLWDQSLKP	1/5
38	^{105}EDIISLWDQSLKPCV	1/5
39	^{107}IISLWDQSLKPCVKL	2/5
121	^{291}LNESVAINCTRPNNN	1/5
122	^{293}ESVAINCTRPNNNTR	0/5
123	^{295}VAINCTRPNNNTRKS	1/5
124	^{297}INCTRPNNNTRKSIY	2/5
125	^{299}CTRPNNNTRKSIYIG	0/5
126	^{301}RPNNNTRKSIYIGPG	1/5
127	^{303}NNNTRKSIYIGPGRA	1/5
128	^{305}NTRKSIYIGPGRAFH	1/5
129	^{307}RKSIYIGPGRAFHTT	4/5
130	^{309}SIYIGPGRAFHTTGR	4/5
131	^{311}YIGPGRAFHTTGRII	4/5
132	^{313}GPGRAFHTTGRIIGD	4/5
133	^{315}GRAFHTTGRIIGDIR	5/5

The results demonstrate that peptides 30-33 and 129-133 include T cell determinants for HIV_{SF2} gp120.

Example 4

A. General

Batches of RPMI-1640 medium were obtained from the Commonwealth Serum Laboratories ("CSL", Parkville, Victoria, Australia), Gibco Laboratories (Grand Island, NY), Flow Laboratories (North Ryde, Australia), or Hazelton Inc. (Lenexa, KS). Eagle's minimal essential medium (MEM), Dulbecco's modified MEM (DMEM), Monomed serum-free liquid medium, penicillin/streptomycin, gentamicin, kanamycin sulphate, neomycin sulphate/polymyxin B sulphate, L-glutamine, sodium bicarbonate and HEPES buffer were supplied by CSL. Ciprofloxacin was kindly donated by Bayer Australia (Sydney, Australia). "Incomplete" culture medium composition was RPMI-1640 supplemented with L-glutamine (2 mM), sodium bicarbonate (2 g/L), gentamicin (50 μg/mL) and HEPES (5 mM). "Complete" medium was incomplete medium supplemented with 10% human serum.

Autologous serum was obtained after defibrination of 50 to 100 mL freshly-drawn human venous blood by gentle agitation with 3-5 g of sterile acid-washed glass balls (5-8 mm diameter) for at least 10 min. Serum was collected from above PBMC bands after density interface centrifugation. Autologous plasma was collected from above the PBMC band after density interface centrifugation of anticoagulated whole blood. Group AB human plasmas, anticoagulated with either acid-citrate-dextrose or citrate-phosphate-dextrose (ACD, CPD), were a gift from CSL. Plasma was converted into serum by the addition of 10 mM calcium chloride and 1 IU/mL (final) of human thrombin (CSL) to plasma prewarmed to 37°C, followed by vigorous agitation for 5-10 min. The mixture was allowed to stand at room temperature for 60 min and the supernatant serum was collected by centrifugation at an RCF of 20000 at 4°C for 20 min. Sera or plasmas were heat-inactivated at 56°C for 45 min in a water bath.

PBMC were obtained from heparinized or defibrinated whole blood from a healthy adult volunteer donor panel. Alternatively, screened buffy coats from blood anticoagulated with CPD, kindly supplied by the Red Cross Blood Bank, Melbourne, Victoria, were used. Blood was diluted slightly to between 1:1 and 2.5:1 (50% to 72% (v/v) whole blood) with incomplete medium and underlayed with Ficoll/Paque (Pharmacia LKB, Uppsala, Sweden) in

a 50 mL polypropylene centrifuge tube in a final ratio of 2:1 diluted blood:Ficoll/Paque. A band of PBMC was isolated at the interface by centrifugation at an RCF of 450 for 25 min. When autologous serum was required, it was recovered from above the PBMC band. PBMC from the band were washed twice by centrifugation in "incomplete" medium containing about 10% (v/v) added autologous serum. The first wash was at an RCF of 450 for 15 min to ensure efficient pelleting of cells from the medium containing residual Ficoll/Paque, while the second was at an RCF of 150 for 10 min to minimize platelet and debris contamination. Cells were suspended in complete medium containing autologous serum or pooled human serum and viable cells were counted on a hemocytometer using trypan blue dye exclusion.

Tetanus Toxoid (TT), and Purified Protein Derivative (PPD) from BCG cultures were supplied by CSL, Melbourne, Australia, and were used at 0.1-10 Lf/mL and 0.1-10 μg/mL respectively. TT was dialysed to remove thiomersal preservative before use. Betapropriolactone-inactivated zonally purified A/Shanghai/11/87 (H3N2) influenza virus suspension was also supplied by CSL. Concanavalin A (Sigma, St. Louis, MO) was used at 2-10 μg/mL. Recombinant Herpes Simplex virus type 2 glycoprotein B was kindly supplied by Chiron Corp. (Emeryville, CA).

Single micromolar-scale resin peptides were made on a Milligen 9050 synthesizer (Milligen/Biosearch, Burlington, MA) and purified by reverse-phase HPLC. Multiple peptides were made on polyethylene pins using the Multiple Peptide Synthesis System (Chiron Mimotopes, Clayton, Australia). Solution phase peptides were generated by cleavage from the pin at neutral pH of a lysine-proline ester linker to form a diketopiperazine (cyclic dipeptide) moiety at the carboxy terminus of each peptide.

In the "standard" lymphocyte proliferation assay, PBMC ($2x10^5$ per well) along with antigen or mitogen were added to 96 well U-bottom tissue culture trays (Nunc, Roskilde, Denmark) in a final volume of 200 μL complete medium per well. Cultures were incubated at 37°C in humidified 5% CO_2 in air for various lengths of time. The stated amount (usually 0.25-1.0 μCi) of tritiated thymidine (^{3}H-TdR) (Amersham International, Bucks, UK, or ICN Biomedicals, USA, specific activity 40-60 Ci/mmole) was added and the plates reincubated for the stated time before harvesting the DNA onto glass fibre filtermats and counting in an LKB 1205 Betaplate liquid scintillation counter (LKB, Turku, Finland).

V-bottom 96 well microtiter culture trays were from Greiner GmbH, Nurtingen, Germany. Flat-bottom 96 well microtiter culture trays were from Nunc, Roskilde, Denmark.

Tests were performed in as many replicates as the cell yield and size of the blood donation from each donor allowed. A minimum of 12 replicates per experimental group was generally used. Results are expressed as: the mean ± SD of the cpm of the replicates; as stimulation indices (mean cpm of stimulated cultures/mean cpm of Cells Alone controls); or the replicates were scored as positive (responding) or negative (nonresponding) based on a cutoff value for cpm of the incorporated ^{3}H-TdR. For the calculation of the cutoff, data from all experimental groups was pooled for analysis.

B. Results

Seven lots of RPMI-1640 medium, and two other base media (MEM and DMEM), were used to make up "complete medium" without antibiotics, supplemented with identical additives including 10% (v/v) of a single batch of pooled human serum. They were then tested in proliferation assays with PBMC from one donor, in response to antigenic stimulation with PPD (Table 1). The characteristics sought were congruous, low background levels with strong antigen-specific proliferation. RPMI-1640 media tested included five different brands (three liquid and two powdered) and different batches of two brands. We also tested MEM and DMEM. A serum-free medium, "Monomed", was tested without supplementation with serum. We found that some media gave high mean background (cells alone) proliferation, and some gave high frequency occurrence of spontaneous proliferation in the background group (media G and F, Table 1). Some of the background group wells which underwent spontaneous proliferation gave counts as high as the specific responses (data not shown). Brand A liquid medium was found to be superior to all others, and further testing of batches of brand A confirmed its consistent performance (results not shown). MEM and DMEM both supported adequate levels of specific proliferation, but were not the most suitable due to high or uneven backgrounds.

In CSL Monomed serum-free medium, antigen-specific responses were markedly reduced compared to RPMI-1640 containing 10% pooled human serum.

Negative control cultures of PBMC from some donors were found to give unacceptably high frequencies of "spontaneous" proliferation in medium containing the antibiotic

combination penicillin/streptomycin but not in medium containing gentamicin. With some donors, responses to antigen were also heightened in the presence of these antibiotics. Several other antibiotics were then tested for their suitability: gentamicin, kanamycin, neomycin/polymyxin, and ciprofloxacin. Gentamicin was chosen due to its low toxicity and absence of stimulatory effect on controls, as well as its stability during culture.

As shown above, serum is a vital component of media for proliferation assays on PBMC. Human AB sera are widely used for the purpose. However, the selection of reliable sera also requires a screening process, as illustrated by the following data. ACD-anticoagulated human plasmas from blood group AB donors were converted to serum and each separate donation tested as a medium supplement in antigen- and mitogen-driven proliferation of three lots of human PBMC. Serum was graded as suitable for use on two criteria. Firstly, each lot of serum should provide a low, even background with all three test PBMC. Secondly, the serum should provide good growth ($>3\times$ mean of background wells) of both the mitogen and antigen stimulated cultures of at least two of the three lots of PBMC. From this and the results with the other two PBMC, sera 40, 44, 46 and 50 were excluded. Sera 44 and 50 were excluded because they did not support proliferation in the other two PBMC tested. Serum 40 did not support antigen-driven proliferation in any of the PBMC even though it supported mitogen-driven proliferation. Thus, screening human AB sera can be a large undertaking requiring careful evaluation. Commercial human AB sera also need to be tested for this purpose as batches vary enormously in suitability.

Sera which fail the selection criteria ("poor" sera) would still be usable within a serum pool if they simply lack factors present in "good" sera, especially if "good" sera are oversupplied with such components. However, if growth suppressing factors are present in poor serum, no benefit will be gained by mixing with good serum. Three good sera and 3 poor sera were tested separately, and as pools, in antigen-driven proliferation assays with four donors. One of the poor sera, P3, was designated "poor" because it generated frequent spontaneous proliferation in control cultures rather than because of low support for proliferation. When all three good sera were pooled (G1.3), results were similar to those when each was tested separately. The proliferation-supporting capacity of serum P3 was reduced when mixed with the other poor sera (P1.3). Likewise, when the poor sera were

pooled with the good sera the antigen-driven proliferative response was greatly reduced by comparison with the pool of good sera. These results indicate the presence of suppressive factors in certain sera rather than a growth factor deficiency.

Autologous serum from defibrinated blood was found to be at least as good a medium supplement as screened pooled serum. In most cases media containing autologous serum gave higher stimulation indices than media containing pooled serum. This was particularly so for antigen-driven proliferation rather than mitogen-driven proliferation. The practical advantage of using autologous serum is that it requires no prescreening, is very easily obtained in sufficient quantity for the experiment at hand, and requires no additional processing to remove anticoagulants or fibrinogen. Autologous serum from a previous bleed of the same donor can also be used.

Variation in autologous serum concentration had no strong effect on antigen-driven proliferation over the range of 5 to 20% serum. All four concentrations tested supported both whole antigen- and peptide-driven proliferation. There was no consistent trend in the mean cpm of wells scored as negative (background). Likewise, the frequency of positive wells and the mean cpm of positives did not change significantly as serum concentration increased.

The effect of time of incubation prior to pulse labeling on ability to detect specific proliferation was determined using complete medium containing 10% autologous serum, 2×10^5 cells per well and U-bottom wells. An incubation time of 3-5 days gave the clearest differentiation between controls and antigen-stimulated cultures, both for whole antigen and for a peptide. The variation in cpm for control (cells alone) wells increases with time, with the most variation occurring after 5 days incubation.

To examine the variation in negative controls ("Cells Alone" controls) over a range of incubation times, four donors' control PBMC were pulse-labeled after one to eight days incubation, using large numbers of replicates. For the first four days' incubation, the mean thymidine incorporation remained fairly constant, but for the next four days there was a steady rise. The coefficient of variation of the mean cpm also increased remarkably from day 5 onward, due mainly to an increasing frequency of wells undergoing spontaneous proliferation. Based on the data thus obtained, and parallel tests with additional

antigen-driven cultures, four days was chosen as the best incubation time for clear differentiation between control and stimulated cultures in determinant mapping studies using peptides. It is possible that PBMC with very high frequencies of antigen-specific precursors may benefit by use of a shorter incubation time (3 days) and conversely that PBMC with a low precursor frequency may benefit by a longer incubation time (5 days).

To determine if well shape had an effect on PBMC proliferation assays, cells were incubated in either U- bottom or flat bottom wells for a range of times with various antigens. The frequency of positives in the No Antigen (cells alone) control, and the frequency of responses to TT, were largely unaffected by well shape. However, cells responded to a T cell determinant peptide from tetanus toxin (P399) more frequently in the U-bottom wells than in flat bottom wells, at both peptide concentrations, regardless of the time of incubation. This may be due to the greater cell-cell contact afforded in U-bottom compared to flat bottom wells. As expected, similar results were found for V-bottom wells, although the latter were not tested as thoroughly as U- and flat bottom wells.

The effect of initial cell number per well on magnitude of the proliferation was measured at a single time point (7 days) after initiation of the cultures. Medium volume was a constant 200 μL per well. At 1.2×10^4 PBMC per well, no antigen-specific response was discernible, possibly due to insufficient numbers of antigen-specific precursor T helper cells. With increasing cell number up to 4×10^5 cells/well, there was a progressive increase in both the mean antigen-driven response and the background. Higher cell concentrations led to a reduction in antigen-driven proliferation, probably due to depletion of medium components during the seven day incubation period. Therefore, use of cell numbers above about 4×10^5 cells per well may compromise the detection of specific proliferation when long incubation times are used.

The effect of initial cell number per well using a variety of well shapes was also determined at a shorter incubation time. PBMC concentrations 10^6, 3×10^5, and 10^5 cells/mL, added in volumes of 100, 50 and 20 μL of medium were used to initiate cultures with between 5×10^3 cells/well and 10^5 cells/well. An incubation period of 4 days was used. The response was expressed as percentage of test wells scored as positive for proliferation. Use of V-bottom wells gave the highest sensitivity of detection of

antigen-specific responses, allowing responses to be detected at 5×10^3 PBMC per well for this donor. Under these conditions, a comparison of the results shows that specific proliferation in either U-bottom or flat-bottom wells was erratic until cell numbers reached 3×10^4 or 10^5 cells/well respectively. That this was not simply a lack of responsive T cells is shown by the result with the V-bottom wells.

The effects of varying the pulse duration, specific activity, amount, and concentration of the ^{3}H-TdR label on readout of PBMC proliferation assays were examined. Mitogen-stimulated PBMC were initiated at 5000 cells/well and pulse-labeled on day 3. The cells incorporated more label into DNA as the extracellular concentration of ^{3}H-TdR was increased. The amount of label incorporated did not reach saturation within the range of concentrations of thymidine tested, regardless of the specific activity of the thymidine. In both PBMC and a continuous B-cell line, the cpm of incorporated thymidine increased with increasing specific activity, except for the continuous B-cell line at the highest specific activity tested (48 Ci/mmol). Increases were not directly proportional to specific activity. The cells were able to incorporate more total thymidine of low specific activity, indicating they were not flooded with thymidine over the dose range tested.

Time course studies of ^{3}H-TdR uptake in proliferating cultures showed a plateauing of incorporation of thymidine after 6h for both PBMC and a continuous B-cell line. This effect was independent of the specific activity over the range tested, and hence the total thymidine concentration. Further studies indicated that even after incubation overnight (>16 h), no further significant increase in label incorporation occurred.

The incubation time at which maximal proliferation occurs is a factor which should be evaluated in any proliferation assay. Use of a long incubation time prior to pulse labeling of stimulated PBMC could give the impression that low doses of antigen are more effective than high doses, due to the "overgrowth" of cultures under optimal stimulation conditions. Conversely, use of a short incubation time may not allow the amplification mechanism to proceed to the point where the effects of single antigen-specific precursors could be detected. An attempt to overcome this timing difficulty was undertaken by trying to maintain available levels of ^{3}H-TdR in the culture medium. Cells might then accumulate label progressively as

they proliferate up to the time of harvest. In this manner, the total proliferation of the cultures over the entire incubation time could be measured.

A large number of replicates of antigen-stimulated PBMC were initiated. Each day, a proportion of the cultures were pulse labeled 3 h and harvested. Others were pulsed daily for 2, 3, 4 or 5 days, beginning after 3, 4, 5 or 6 days of label-free incubation. Although incorporated thymidine cpm values were noticeably higher for multiple-pulsed cultures than for single-pulsed cultures, the higher background (Cells Alone) cpm meant that there was little difference in stimulation indices and thus no advantage in multiple pulsing.

The increase in cpm between 3 h and 27 h for cultures harvested after a single pulse, regardless of the day on which the first pulse was given, is consistent with the finding that incorporation continues up to 6 h after addition of the thymidine.

It is often convenient to store thymidine-pulsed cultures before harvesting. Replicate cultures were either harvested immediately after the pulse label period, stored up to 24 hrs at 4°C or frozen for various periods. We found no significant differences in the mean cpm or standard deviations. Therefore, if time restrictions or assay size hamper the immediate harvesting of pulsed cultures they can be frozen for storage of indefinite duration, or held at 4°C for 1 day, provided all plates of a single assay are handled identically.

C. Results

The experimental conditions should not only avoid nonspecific stimulation, but also provide an environment in which specifically stimulated cells can proliferate optimally. We have developed methods which do not simply look for the greatest magnitude of thymidine incorporation or stimulation index in PBMC, but rather for the highest sensitivity and reliability in detecting and counting antigen-specific T helper cells.

Previous workers have noted the importance of culture conditions in obtaining reliable, reproducible results. Other studies showed that factors such as cell concentration, incubation period, pulse label parameters and concentration of stimulant had interrelated effects on the measured proliferation rate of PBMC. Those studies concentrated mainly on the effects of mitogen or allogeneic stimulation. E.J. Hensen *et al.*, Hum Immunol (1984) 10:95 noted the importance of using a large number of replicates to accurately measure the response to a single antigen. Importantly, Hensen *et al.* emphasized the effects of random proliferation

occurring in control cultures, and the inadequacy of the stimulation index as a measure of specific proliferation.

Our results showed that medium-related factors which affected the frequency of non-antigen-specific positives in the Cells Alone control were the base medium, antibiotic, and serum. Batches of RPMI-1640 medium from different manufacturers varied remarkably in quality, not only in ability to support growth, but also in their tendency to give rise to antigen-nonspecific proliferation. P.T.A. Schellekens *et al.*, Clin Exp Immunol (1968) 3:571 noted, with PHA-stimulated cultures, that the results were not dependent on the choice of base medium, in agreement with our contention that culture conditions in antigen-driven proliferation are more critical than in mitogen-driven proliferation. RPMI-1640 is used because it was specifically designed for human lymphocyte culture. High backgrounds created by media components may be due in part to the presence of various levels of B-cell-stimulatory endotoxins, or contaminating traces of T-cell-stimulatory substances such as bacterial superantigens.

Although most cell culture texts advise the use of penicillin and streptomycin (P/S), we have occasionally found this antibiotic combination unsuitable. Negative control cultures of PBMC from some donors gave unexpectedly high frequencies of "spontaneous" proliferation in medium containing P/S. With these donors, the response to antigen was also heightened. The spontaneous proliferation may be due to penicillin, to which many people are allergic, however this has not been proven. Gentamicin was found to be free of such problems and was used routinely.

Serum is the most important single factor in the success of proliferation assays. The detrimental effects of using stored, unscreened serum as well as the differing effects of different serum sources on either antigen or mitogen stimulation have been noted by others bovine serum and other heterologous sera enhance background proliferation in human PBMC assays to levels which can obscure antigen-specific responses, whereas selected human sera do not. In contrast, foetal bovine serum has also been found to be inhibitory to human PBMC proliferation in whole blood culture systems. Despite reports of serum-free media for culture of human PBMC, there has been no general acceptance that serum-free media will

support efficient proliferation of human PBMC, and we also found that serum supplementation was needed for the serum-free medium formulations we tried.

Selection of suitable human sera is best performed using PBMC of several donors whose responses to certain antigens is already well characterized. Sera supporting strong specific proliferation and low backgrounds can then be pooled, aliquotted and stored at -20°C or colder. An attractive alternative to screened human serum is autologous serum. PBMC and serum can be recovered from the same whole blood sample in high yield by defibrination and dilution of blood with RPMI-1640 medium, followed by density interface centrifugation as described. This process provides more autologous serum than required for a 10% (v/v) final concentration in culture medium at the cell densities used. We found autologous serum to be at least as good as screened pooled human serum. Autologous serum can be heat-inactivated at 56°C for 45 min without loss of growth-supporting qualities.

Our data show that it is unwise to mix poor sera with good sera in an effort to make the supply of serum last longer. The inhibitory components in serum are yet to be defined. It has been reported that high concentrations of certain complement components in some sera inhibit PBMC proliferation. Best use of good sera can be obtained by using them at 5% (v/v).

The incubation time and the number of PBMC added per well are interrelated factors which must be adjusted together. If input cell concentration is too high, proliferation at long incubation times can be dramatically reduced, possibly by exhaustion of the nutrient or buffering capacity of the medium, or by inhibition of cells due to high cell densities. Use of too few cells per well requires very high numbers of wells to allow detection of antigen-specific precursors at biologically significant frequencies (*e.g.* >1 per million PBMC). Short incubation times (4 days) give sensitive detection of positives with low and consistent backgrounds.

Maximum sensitivity of detection of proliferation is vital to calculation of accurate antigen-specific precursor frequency when limiting dilution data is sought. The optimal time for incubation of antigen-driven PBMC proliferation assays using the methods described is 4 days, whereas 2 to 3 days is optimal for mitogen-driven cultures.

Cell numbers per well and well shape are also interrelated factors in antigen-driven PBMC proliferation. The use of round-bottom wells to improve cell-cell contact allowed the use of reduced cell numbers in proliferation experiments. Use of V-bottom wells, although useful for improving sensitivity, should be avoided if high cell numbers (>100,000 cells/well) are to be used, as responses can be inhibited under these conditions.

Exogenous thymidine is quickly assimilated into the intracellular pool and used for DNA synthesis during the S phase of the cell cycle. Trace-labeling can be used to measure rates of DNA synthesis, provided the thymidine is of low specific activity, <2 Ci/mmol. However, use of low specific activity thymidine can lead to addition of excess total thymidine, changing the conditions to flood labeling. Use of high specific activity thymidine (40-80 Ci/mmol) results in cytotoxicity, probably due to radiological damage. We chose to use trace labeling with a low dose of high specific activity thymidine since the cultures are terminated after a short labeling time (6 h) and the intent is primarily to detect significant differences between proliferating and nonproliferating cultures. As thymidine is sold by radioactive content, a small dose of high specific activity is the most economical way to label. Under these conditions, the dose of thymidine is limiting and incorporated cpm are thus proportional to the rate of DNA synthesis at the time of addition of the label.

As may be expected from the shape of the curve of incorporated thymidine against time, the use of multiple small doses of thymidine over several days did not significantly enhance the total incorporation of thymidine by comparison with a single dose incubated for 27 h, and any advantage in total incorporation by antigen-stimulated cultures was lost due to increased incorporation in the unstimulated controls.

We often find it convenient to store assay plates after the pulse label period. Plates may be placed at 4°C overnight or frozen at -20°C for several days without apparent loss or breakdown of the DNA. Plates may be then processed for harvest of DNA and counting of thymidine as time permits.

Although it is wise to test parameters for every PBMC/antigen combination, the following recommendations apply in most cases.

1. Use screened HEPES-buffered RPMI-1640 with 10% autologous serum or screened, pooled human serum, adding gentamicin if an antibiotic is required,

2. Culture up to 2 × 10^5 PBMC/well in multiple wells (8 or more) in U-bottom microtiter plates or up to 10^5 PBMC/well in V-bottom plates for 4 days,
3. Trace label with 0.25uCi/well of high specific activity (40-80Ci/mmol) 3H-TdR for the final 6h of the incubation period prior to harvesting the DNA onto glass fibre filter mats and scintillation counting.
4. Analyse the results using an algorithm which takes account of the possible presence of responding wells in the Cells Alone nonresponding wells (wells lacking antigen-specific precursors) among the wells to which the test antigen has been added.

Example 5

A. Procedure

Overlapping dodecapeptides for determinant scanning were synthesized with termini consisting of a carboxy-terminal beta-amino-alanine-diketopiperazine (β-dkp) group and an acetylated amino terminus. The multipin peptide synthesis strategy was used. Work with several clonal T cell systems has shown that N- and C-terminal-blocked peptides are as efficient or more efficient in T helper cell activation than unblocked peptides, in contrast to cytotoxic T cells. Peptides were cleaved into sterile 0.1 M sodium bicarbonate in 96-well microtitre trays. The purity of representative peptides was assessed using HPLC and was found to be generally >80%. Wells were found to contain an average of 10 nmol cleaved peptide by amino acid analysis.

Bulk peptides:

P399 (Ac-QEIYMQHTYPIS-b-dkp, tt 257-268), P442 (H-EQDPSGATTKSAM-LTNLIIFGPGPVLNKNEV-OH, tt 141-171), P443 (H-SVDDALINSTKIYSY-FPSVISKVNQGAQGIL-OH, tt 581-611), P444 (H-DTQSKNILMQYIKANSKFI-GITELKKLESKI-OH, tt 821-851), P445 (H-IEYNDMFNNFTVSFWLRVPKVS-ASHLEQYGT-OH, tt 941-971), P459 (Ac-VRDIIDDFTNESSQKT-NH2, tt 616-631) and P480 (H-FNNFTVSFWLRVPKVSASHLE-OH, tt 947-967) were prepared by solid phase peptide synthesis using an Applied Biosystems 430A peptide sythesizer. Peptides were

purified to >75% and their compositions were confirmed by amino acid analysis. TT was a gift from the Commonwealth Serum Laboratories, Melbourne, Australia.

PBMC were from heparinized venous blood of healthy volunteers who had been routinely immunized with TT. PBMC were isolated by density-interface centrifugation over Ficoll-Paque (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) as described (D.A. Mutch *et al.*, Pept Res (1991) 4:132-37. The PBMC were resuspended in complete medium for counting. The average yield of PBMC from whole blood was 2×10^6/mL with a range of 1.2×10^6/mL to 2.9×10^6/mL.

Peptide-stimulated proliferation assays using 2×10^5 PBMC per well were performed in 96-well round bottom tissue culture grade mictrotitre plates (Nunc, Roskilde, Denmark). Antigens were added in 20 μL of 0.1 M sodium bicarbonate to give a final volume of 200 μL per well. Because PBMC often exhibited a low frequency of T cells specific for particular determinants, all assays were carried out using at least 16 replicates per test. PBMC were incubated at 37°C in 5% CO_2 in humidified air. After 138±2 h, proliferation was detected by pulsing with 0.5 μCi tritiated (methyl-^{3}H) thymidine, ^{3}H-TdR, (40-60 Ci/mmole, Amersham Australia, Sydney) per well for 6 h. DNA was harvested onto glass fibre filter mats (Skatron, Sterling, VA, USA) and incorporated thymidine was measured in an LKB 1205 Betaplate liquid scintillation counter. All assays included at least 16 wells each of negative controls (20 μL of 0.1 M sodium bicarbonate instead of peptide solution) and positive controls (TT at 1.0 Lf/mL and 0.1 Lf/mL, also in 0.1 M sodium bicarbonate buffer).

The restimulation assay was that of E.J. Hensen *et al.*, Human Immunol (1984) 10:95 modified for use with peptides. For each donor, aliquots of PBMC at 2×10^6/mL in three glass petri dishes were stimulated with TT (1.0 LF/mL), P399 (1.0 μg/mL) or no antigen respectively. After 6 days, cells were washed to remove any residual peptide or antigen, and resuspended in the same volume of fresh complete medium. To test for strength of proliferation at this time, aliquots of 100 μL were pulse-labeled using ^{3}H-TdR. Three days later, further 100 μL aliquots was tested by the same method to see if proliferation had subsided. The cells from the three petri dishes were then washed and resuspended at a concentration of 2×10^5/mL. Replicate aliquots of 100 μL from each of the three groups were

dispensed into wells of U-bottom microtitre trays for restimulation with the following antigens: P399 at 10, 1.0 and 0.1 μg/mL, TT at 1.0 and 0.1 Lf/mL, or no antigen. To ensure adequate numbers of APC were available, wells were supplemented with 1×10^5 gamma-irradiated (3000 Rad) autologous PBMC. After 3 days restimulation (day 12), cells were examined for proliferation by pulsing with ^{3}H-TdR for 6 hours.

Four regions of the tt sequence were studied. Three of these regions, corresponding to peptide pools spanning residues tt 141-171, 581-611 and 821-851 were stimulatory regions common to the majority of donors tested. The fourth region, tt 941-971, homologous with a reported universally immunogenic T cell determinant (tt 947-967) was chosen because the pooled 12mers were not stimulatory for any of the donors tested. This was therefore a test of the dependence, on peptide length, of stimulation by peptides from a known stimulatory sequence. PBMC were stimulated with pooled 12mers or with the corresponding 31mer peptide containing all the residues of the individual peptide pool. Responses were compared using 32 replicates per test to enable differences in frequencies of responses to be distinguished. The 31mer peptides were tested at a range of concentrations whereas the peptide pool was tested at a single concentration, comprising 0.3 μM of each peptide.

Starting from the assumption that the cpm data for negatives (nonresponding cultures) would be approximately normally distributed about the background mean, the lowest values were used for calculation of a mean and standard deviation. A temporary cutoff of the mean plus three times the standard deviation was calculated, and all cpm values below this cutoff were used for calculation of a new temporary cutoff by the same process. This process was reiterated until no further cpm values were incorporated into the calculation, *i.e.*, until the cutoff remained stable.

After each well was scored as negative or positive, Poisson statistics, which are appropriate to low frequency events, were used to determine whether any difference between the negative control (cells alone) group and each experimental group was significant. Only data significant at the 5% or better ($p < 0.05$) level was used.

B. Results

Development of the method for T cell determinant mapping using PBMC

The parameters of the PBMC proliferation test were determined using several donor/whole protein antigen combinations, and were later retested with a selection of peptide determinants found during the course of this work. All the present work used a six day incubation period, chosen because proliferation usually peaked at that time. In later work, we have opted for a four day incubation to achieve the best discrimination between controls and responding cultures.

The need for high numbers of replicates ruled out dose-response titrations on PBMC with each of the large number of peptides used in an initial scan. A target concentration of 0.3 μM of each peptide therefore was chosen, based on the limited amount of peptide available and because most T helper cell clones respond to that concentration, even though it may not be the optimum. A reported T cell determinant, was recognized by the majority of TT-responsive donors. The higher concentration of peptide (2.4 μM) stimulated a higher frequency of positive responses from PBMC than the lower concentration. The use of 0.3 μM of a single peptide in a stimulation test could thus lead to failure to detect low frequency responses when they are present. This problem should be lessened when pools of overlapping short peptides, offset in start position by one residue, are used.

The choice of 12mers as the peptide length for high-resolution scanning was based on a length of 9 to 11 residues for many known T helper cell determinants and on the finding that 12mers were sufficient to detect all major determinants of hen egg lysozyme. For the scanning of large proteins with PBMC, the individual testing of every possible overlapping 12mer becomes completely impractical, and thus a pooling/decoding strategy was devised. It was found that up to 30 peptides could be pooled for an initial test without compromising the response to individual peptides. Modeling of various ways in which peptides could be grouped revealed that pooling of contiguous overlapping peptides gives the most useful information.

The PBMC determinant scanning method was developed in studies with tt. Initially, a panel of 12mer peptides homologous with the tt sequence as likely T cell determinants was chosen. One peptide, P399, was found to give proliferation with PBMC of ten out of seventeen TT-responsive donors tested and was therefore chosen for a specificity test.

To see if proliferative responses observed in individual peptide-stimulated PBMC cultures were due to peptide-specific T cells, and to see if these cells were also able to respond to the whole antigen from which the peptide was derived, a sensitization-restimulation assay was carried out. PBMC were stimulated with either peptide P399 (1.0 μg/mL) or TT (1.0 Lf/ml). Unstimulated control cells were incubated without added antigen under the same conditions. After 6 days in culture, proliferation of antigen-stimulated cultures was demonstrated. All three groups of cells were washed to remove lymphokines and any antigen still present in the medium. On day 9, proliferation had subsided and the PBMC were washed again. Each culture was then divided into six treatment groups and restimulated (P399 at 10, 1.0 and 0.1 μg/mL, TT at 1.0 and 0.1 Lf/ml, and No Antigen control) in triplicate cultures. To ensure that sufficient APC were available, 10^5 gamma-irradiated autologous PBMC were added to each culture. Proliferation was measured three days after restimulation.

The results demonstrated that peptide-stimulated PBMC cultures are able to be restimulated in a dose-dependent fashion by both the sensitizing peptide P399, and the whole antigen TT. Control incubated PBMC not previously stimulated with either antigen showed little proliferation, in the short time frame and with the low number of input unirradiated PBMC, to either the peptide or the whole antigen. This demonstrates that peptide-stimulated cultures could be reproducibly restimulated by the same peptide and were not responding to undefined components of the culture medium. They were also restimulated by whole TT showing that they were specific for the antigen from which the peptide was derived. In addition, the TT-sensitized PBMC were restimulated by both P399 and TT. Thus, in this instance the cells responding to TT must have included a high proportion of P399-responsive cells also.

To compare the effectiveness of pools of short peptides with that of single long peptides, it is necessary to work with antigen/donor combinations where specific helper T cells are known to exist. Responses to three dominant determinant regions within the TT sequence found using peptide pools were compared to responses obtained using 31mer peptides that contain all the residues encompassed by that particular peptide pool. A fourth region spanning a published "promiscuous" T helper cell determinant but found to be nonstimulatory

using pools of peptides, was included to see if a single 31mer could detect a determinant where the pooling method had failed to do so. The long peptides were tested over a wide range of concentrations so that there was no bias against the long peptides due to suboptimal concentrations being used. In a pool of 12mer peptides, the effective concentration of stimulatory peptide will depend on the length of the determinant, as those shorter than 12 residues may be represented in two, three or more overlapping peptides.

Although peptide pools 30 and 42 induced proliferation of PBMC at 0.3 μM/peptide, the corresponding 31mer peptides P443 and P444 stimulated few or no responses at any concentration tested. Thus, of the three major determinant regions detected by pools of short peptides, P442 was the only one of the corresponding 31mer peptides able to induce comparable proliferation. Even then, the proliferative responses to P442 occurred at lower frequency than for the peptide pool. The results also demonstrated that three donors tested were unresponsive to pool 48 or the 31mer peptide P445, which contains the known T cell determinant TT 947-967, despite being responsive to the peptide corresponding exactly to the published T cell determinant, tt947-967. Thus, for this determinant, neither the longer nor shorter peptides are efficient stimulators.

The most efficient peptide length for specific stimulation of PBMC is unknown. Factors such as the most efficient length for uptake by MHC class II, small differences in peptide sequences seen by similar T helper clones, and the necessity or otherwise of processing of a peptide before it can be presented will affect the outcome of a stimulation test with pooled peptides on polyclonal T cells (PBMC).

To investigate this problem, four sets of overlapping peptides of different lengths: 10, 12, 14 and 16 residues, spanning a known T helper cell determinant-containing region of tetanus toxin (INSTKIYSYFPSVISKVNQGA; tt 587-609), were synthesized. Peptides of each length were then used to make up three separate pools, each pool containing peptides offset by 1, 2 or 3 residues in their "start" (*i.e.*, N-terminal) residues. The results demonstrated that as the offset increases, the frequency of positive responses decreases, as would be expected if not only length but also "frame" of the determinant within the peptide were important. For pools of shorter peptides (10 and 12mers) this decrease was so dramatic that for peptides offset by 3, no proliferative responses were observed. This suggests that none

of the peptides in the pools offset by 3 contained the stimulatory sequence. In contrast, for 14mer and 16mer peptides, the pools offset by 2 were as effective as those offset by 1. In addition, significant responses were obtained using 14mer and 16mer peptides offset by 3.

These results show that as the length of the peptides was increased from 12 to 16 residues, the frequency of positive responses also increased. A repeat of part of this assay with another sample of the same lot of cells (frozen PBMC) confirmed these results, although there was a slight variation in test conditions because the peptides had been diluted in complete medium containing 0.2% acetonitrile. Other experiments revealed that addition of up to 2% acetonitrile to the culture medium did not affect proliferation tests on PBMC.

We sought to combine the use of short synthetic peptides, which require little or no processing to be active in T helper cell assays, with the use of unselected PBMC as the source of polyclonal T cells. Using PBMC, the repertoire being examined will not be biased by prior in vitro selection of the best-growing or most frequent clones. We therefore had to solve the combined problem of the limitation in the number of PBMC available from any one donor, and the relatively low frequency of T cells specific to any single determinant. This led us to devise a practical approach involving pools of short peptides and large numbers of replicate cultures.

There are several important limitations when applying such methods to thorough screening with PBMC. Depending on culture conditions, the most important one being the culture medium, a significant frequency of "false" positives may occur in the unstimulated control cultures. We have minimized the effect of this factor in three ways. Firstly, media components other than serum have been thoroughly screened to ensure the lowest background stimulation, while still supporting strong antigen-driven proliferation. Secondly, the number of replicates of unstimulated controls is made as large as practicable. The statistical test for a difference between this control group and any test group increases in accuracy with increasing numbers of replicates. Thus, detecting a difference when one is present is more likely, and the estimate of the magnitude of the difference has a higher reliability, as the number of replicates is increased. Thirdly, calculation of a cutoff cpm value to determine the threshold for scoring cultures as positive is based on an method described above. The tests for specificity demonstrated that TT must be processed by these particular donors in

such a way that the sequence contained within P399 is generated and bound to MHC class II molecules for presentation to P399-specific T cells. Not only were TT-sensitized PBMC able to respond to restimulation by P399, but P399-sensitized PBMC were also able to react to the whole antigen, TT. Thus, the response of PBMC to the peptide was unlikely to be due to fortuitous crossreactivity with T cells primed by a different antigen.

The test for efficiency of pooled short peptides by comparison with the corresponding 31mers showed that the former could be more efficient. This agrees with tests on mouse T helper clones exposed to determinants in the context of longer peptides containing added nonhomologous sequences. These results suggest that there can be a block to recognition of long synthetic peptides by helper T cells. There are many reasons why this may occur. Long peptides may require uptake and processing by specific pathways, compared with smaller synthetic peptides which can be presented without processing. Antigen processing and determinant formation by APC has been shown to vary even for donors with the same restriction element or recombinant mice of identical MHC haplotype, which may be due to a requirement for specific protease(s) for generation of particular determinants.

The findings suggest that within the length range of 12 to 16 residues, longer peptides are more efficient for detecting T helper cell determinants. It is not necessary to test pools containing every overlapping peptide along a sequence, if pools containing peptides offset by two residues give an equivalent result. The use of peptides offset by two is advantageous because it significantly reduces the number of peptides required for systematic synthesis and testing of a given protein sequence. Peptides in the 13 to 18 residue range have been found by extraction from purified class II molecules, so it appears that synthetic peptides of length similar to the native peptides are the most efficient.

Example 6

The method of Example 5 can be applied to any antigen of known sequence to which human or animal subjects have a measurable T helper response. We chose to study tetanus toxin (tt) because it is a commonly used human immunogen in which very high frequencies of specific responding T helper cells occur. Considerable determinant mapping of tt with human T helper cell clones has been reported, but a limitation of methods used for initial

location of determinant regions was that they relied on efficient processing of protein fragments by pathways similar to those operating with the whole antigen. It has been shown that cells deficient in specific enzymes can fail to process and present a particular peptide despite normal ability to process and present whole antigen or other peptides.

Using pin technology (N.J. Maeji *et al.*, J Immunol Meth (1990) 134:23), we synthesized the set of 1304 overlapping dodecapeptide sequences spanning the entire 1315 residues of the tt sequence and cleaved them into a physiologically compatible buffer ready for testing. PBMC from donors shown to respond to tetanus toxoid (TT) *in vitro* were screened against peptide pools to locate all determinants in the sequence. Pools were used to keep testing to a manageable, realistic scale. PBMC were chosen as the source of responsive T helper cells because they have the advantage of providing a repertoire unbiased by in vitro selection of the best growing or most prevalent clones. When using PBMC, however, large numbers of replicates and controls must be included to ensure statistically significant results are obtained.

Several stimulatory pools were identified which were common to many donors. T helper cell determinants within such stimulatory pools were then precisely located for several donors by testing the individual peptides comprising each pool. The data thus obtained is more relevant to the total response of human T helper cells to TT than data obtained by investigation of limited portions of the antigen. Three of the five T cell determinants common to the donors tested have not previously been reported.

A set of 1304 overlapping 12mer peptides spanning the tt sequence (U. Eisel *et al.*, EMBO J (1986) 5:2495, incorporated herein by reference) and offset by one residue was synthesized. The multipin peptide synthesis system, which results in non-toxic peptide solutions ready for use in bioassays, was used. As it was impractical to screen each peptide separately for its ability to cause proliferation of PBMC, we used a peptide pooling strategy to identify regions along the sequence containing T helper cell determinants.

We chose to screen peptides as sixty-six pools of approximately 20 sequential overlapping peptides each. The size of the pools was selected so that the size of both the initial scan and the subsequent "decodes" of stimulatory pools would be manageable. As only those peptide pools stimulating proliferative responses in the first round of testing require "decoding", the number of subsequent decodes reduces with increasing number of nonstimulatory pools. It

should be noted that the peptides from the amino-terminal end of a pool overlap with the preceding pool and likewise the peptides from the carboxy-terminal end of a pool overlap with the following pool.

The concentration of each peptide used in the final culture was 0.3 μM, which was less than the estimated peptide concentration required to approach optimum stimulation (estimated to be about 1 μM). The choice of final peptide concentration was constrained by the need to keep the volume of peptide solution to $<10\%$ of the culture medium to avoid dilution and possible toxicity effects, and also by the peptide concentration (60 μM) of the stock solutions. It should be emphasized that determinants of less than 12 residues in length will be present in 2, 3 or more overlapping peptides in the pool, and therefore the effective concentration of shorter determinants will be higher than that of longer determinants.

PBMC from nine HLA-typed donors known to respond to TT *in vitro* were initially scanned for their ability to respond to each of the 66 peptide pools. Results are only shown for pools which stimulated a significantly higher number of wells than the cells alone control ($p<0.05$). Where only one well in a test group showed proliferation, even where this was significantly higher ($p<0.05$) than the cells alone control, we chose to treat this as not significant in the sense of representing a determinant region of tt. Therefore, such single positive wells were not counted in the summary of donors responding to that pool.

The results showed that many pools stimulated PBMC from more than one donor. Major areas of reactivity, to which more than half of the donors responded, were Pools 30 and 42. A further five pools stimulated four of the nine samples of PBMC. All donors, with the exception of donor "C" responded to a pool unique for that donor while 17/66 (26%) of the pools were not stimulatory for any donor.

The individual peptides within four stimulatory pools were tested to identify the individual peptide(s) responsible for proliferative responses incurred by the pool. This test is termed a "decode". Single peptides were tested at 1 μM, approximately three times the concentration of individual peptides used in the pool. The purpose of this was to use a concentration close to the effective concentration which occurs when more than one peptide within a pool contains a determinant. That is, because the length of determinants found with T helper cell clones has been eight or nine amino acids, stimulatory sequences of this length would be

present in four or five different overlapping peptides within the pool, making the effective concentration 1.2 μM or 1.5 μM respectively.

The two most common stimulatory pools, 30 and 42, were decoded. This enabled us to find out whether published T helper cell determinants could be precisely identified using this method. Peptides within pool 30 contain sequence YSYFPSVI (tt 593-600), the determinant for a human tt-specific T helper cell clone. Pool 42 spans sequence QYIKANSKFIGITEL (tt 830-844), reported to contain a "universally immunogenic" DR-restricted determinant.

Decoding of pool 30 showed that five overlapping 12mers with start residues 589 to 593 were stimulatory for at least one of the four donors. These 12mers all contain the sequence YSYFPSVI, identical to the published determinant. In this case, single positive wells were regarded as meaningful because low frequency positive responses appeared to be clustered, *i.e.*, occurred with peptides related by having a high degree of overlap (shared sequence).

Decoding of pool 42 showed eight successive 12mers, with start residues 826 to 833, capable of stimulating PBMC of at least two of the four donors. All these peptides overlap the core of five residues, KANSK, within the reported determinant, tt 830-844.

As the region tt 579-689 (pools 30 to 34) consisted of five commonly stimulatory pools, we chose to decode two additional pools within this region to identify determinants not previously reported. Testing of individual peptides within pool 31 and the first two peptides of pool 32 revealed a series of six overlapping stimulatory 12mers with start residues 616 to 620. All these peptides contain the 7mer core sequence IDDFTNE (tt 620-626).

Decoding of pool 33 showed that the response to this pool was due to two distinct determinant regions. The T helper cell determinants within this region were centred on sequences IVPYIGPA (tt 642-649) and KQGYEGNFI (tt 654-662) respectively.

To see if these findings using 12mer peptides would also apply for longer peptides, we synthesized a 16mer which encompassed the "envelope" sequence of the stimulatory peptides from pool 32 (residues 616 to 631; referred to as peptide P459). P459 was tested at two concentrations; high (10 μM or 5 μM) and low (1 μM), using 8 to 32 replicates per concentration depending on the number of PBMC available. The results deomonstrated that PBMC from the four donors who had responded to the pooled peptides also responded to P459. Of the 11 donors randomly selected, 10 responded to at least one concentration of

P459, suggesting that these responses were probably not restricted to a single HLA Class II allotype.

To see if a cocktail of dominant determinants of an antigen could stimulate responses as strong or as frequent as responses to the whole antigen, the available information on tt determinants was utilized. Peptides containing five T helper cell determinants of tt, including determinants previously reported and from the present work, were pooled and the cocktail was tested in parallel with TT.

A comparison of the frequencies of proliferative responses incurred by the two antigen preparations using PBMC from thirteen donors is shown in. The pool and TT were each tested at four concentrations using 8 replicates per concentration. The frequencies of proliferative responses incurred by the pool are generally lower than those incurred by TT.

We have demonstrated that stimulation of human PBMC with pools of short synthetic peptides, followed by decoding to single stimulatory peptides, is a practical way to exhaustively map T helper cell determinants of entire protein sequences. Five major T cell determinant regions along the tt sequence, which appeared not to be restricted to a single MHC class II allotype, were identified. Two of these regions correspond to published T helper cell determinants whereas three have not previously been reported.

The peptide pooling strategy for T cell determinant identification has major advantages over the use of protein cleavage fragments or use of long synthetic peptides with small overlaps. All overlapping peptides of a length within the range of naturally processed peptides (13 to 18 residues) can be synthesized without stretching the resources of most research groups. With the pooling/decoding approach, the task of testing all these short peptides of an antigen on PBMC of individual donors is achievable.

In this work, the choice of 12mers as the peptide length for testing sequences homologous with tt was based on known minimal determinants and experience with mapping of determinants with polyclonal T cells from mice, prior to information on the length of naturally-processed Class II-associated peptides becoming available. Even though the 12mers detected many previously unknown determinants, had we used longer peptides or more donors we may have detected even more determinant regions. Thus, the scanning reported here, while more thorough than any previously reported, probably does not represent the

total spectrum of determinants for tt because it does not include donors of all MHC types tested at all peptide lengths. While carrying out the tt determinant scanning reported here using every overlapping 12mer, we found that pools of slightly longer overlapping peptides (14 to 16 residues in length) offset by two or three residues are also effective. The number of peptides needed can thereby be significantly reduced, although there is usually a small reduction in sensitivity of detection of peptide-responsive cells when peptides offset by more than one residue are used. A reduction in sensitivity in these circumstances may be due to a decrease in the effective concentration of stimulatory sequences (since each determinant is represented in fewer peptides), or due to non-optimal N- and C-terminal residues in the peptides used.

The "decoding" of adjacent peptide pools showing significant stimulation of PBMC can distinguish between two possibilities. The stimulatory sequence(s) in successive positive pools of overlapping peptides could occur at the boundaries of the pools. Both pools may then contain the same stimulatory sequence, despite the impression that two determinants are involved (donors B and D responding to pools 31 and 32). Alternatively, donors responding to adjacent pools may be responding to independent, unrelated determinants separated by nonstimulatory sequences (donor B responding to pools 30 and 31).

Within the four pools decoded, there were cases where at least four overlapping 12mer peptides were stimulatory. This may be due to a minimal determinant of 9 residues shared among all four 12mers, but because the PBMC system is polyclonal, these peptides could be activating the progeny of more than one T cell clone. The proliferative response to related peptides could thus be due to activation of clonal progeny of one precursor T cell by a sequence common to the peptides, or due to activation of a number of independent T cell clones able to respond to similar (but different) sequences within this region. A test of these alternatives would be to use peptides of the minimum length needed to bind to MHC Class II molecules and stimulate T helper cells. This would enable the effect of small differences in recognised sequences on the measured frequency of specific clonal progeny in PBMC to be seen.

Contrary to first appearances, the physical length of our nominal "12mer" stimulatory peptides is consistent with the 13 to 18 residue length range of the peptides bound to Class II

antigens. This is because our peptides all consist effectively of 15 residues, having 12 residues of the tt sequence with a constant tripeptide moiety (b-dkp) at the carboxy-terminal end. We have found that identical 12mer peptide sequences lacking the b-dkp group (*i.e.*, with free carboxy-termini) are less effective at stimulating PBMC. An acetylated amino terminus can also lead to increased effectiveness of T helper determinant peptides. T helper cell clones can be stimulated by b-dkp bearing peptides of 8, 9 or 10 residues, suggesting that peptides containing 12 residues of the antigen sequence have more than the required amount of sequence needed to allow MHC class II binding and recognition by the helper T cell receptor.

The finding that the amino terminus of the peptide is an important and consistent part of the peptide that binds to MHC Class II antigens suggests that peptides differing in amino-terminal position by only one residue would activate different populations of T helper cells. If this is the case, then testing smaller numbers of longer peptides could result in failure to detect some determinants, since peptides with the required N-terminal residues may not be present in the pool. We have found that a series of contiguous overlapping peptides from within stimulatory pools are stimulatory, suggesting that the N-terminal residue is not critical in determinant mapping with PBMC.

APC play a critical role in antigen-stimulated PBMC proliferation assays. Short synthetic peptides can be efficiently presented by a range of APC, including B cells, monocytes, dendritic cells and possibly other APC. It is likely that efficient uptake and presentation of 12mer peptides by APC in PBMC occurs in our experimental conditions, because addition of adherent cells from autologous PBMC does not cause an increase in the frequency of positive responses. It is known that short peptides can be taken up directly by MHC class II molecules without being processed, but the relative significance of this pathway versus an intracellular pathway for peptides interacting with APC in PBMC is unknown at this time. For longer peptides, however, inefficient detection of precursor T cells may be occurring, since certain pools of 12mers were stimulatory for PBMC in contrast to 31mer peptides spanning the same sequences as the stimulatory pools.

One valuable consequence of the present work is that it shows how all the T helper cell determinants of an antigen could be identified by an unbiased method, leading to a greater

understanding of many aspects of the immune system, including the basis of determinant selection and the factors in peptide sequences underlying MHC Class II restriction. This may enable accurate prediction of T helper cell determinants from primary sequence data alone.

Knowledge of the T cell determinants of an antigen will allow design of reagents for immunization or immunosuppression. Peptides containing T cell determinants may be able to be used alone or in combination with whole antigens to increase the immunogenicity of vaccines. A peptide which helps the formation of IgG rather than IgE may alleviate an allergy. Alternatively, a determinant responsible for an autoimmune disease could be modified to lead to tolerance and therefore alleviation of the disease.

Pools of T cell determinants may also be used as effective substitutes for whole antigens in diagnostic proliferation assays. Testing of the pool of peptides containing five human tetanus toxin T helper cell determinants showed that all donors tested recognized at least one determinant within the pool. This pool of peptides thus represents a reproducible T cell stimulatory antigen mixture which may help the standardization of T cell proliferation tests worldwide.

WHAT IS CLAIMED:

1. A T cell epitope peptide selected from the group consisting of TENFNMWKNNMVEQM, NFNMWKNNMVEQMQE, NMWKNNMVEQMQEDI, RKSIYIGPGRAFHTT, SIYIGPGRAFHTTGR, YIGPGRAFHTTGRII, GPGRAFHTTGRIIGD, GRAFHTTGRIIGDIR, GKNLPVLDQL, IAWYRMGDNCAIPITV, AGTYLRLV, LTSKAYQQG, CPIRTQPRWSYYDSF, INDWTEITQFILE, PRWPRWSYYDSFSAVSEDNLGFLMHAPAFETAGTYLRLVKINDWTEITQFILEHRARASCKYAL, ALYSLKIAGWHGPKP, APAAPSNPG, YSYFPSVI, KANSK, IDDFTNE, IVPYIGPA, KQGYEGNFI, EQDPSGATTKSAMLTNLIIFGPGPVLNKNEV, and QEIYMQHTYPIS.

2. A T cell epitope peptide comprising a sequence of at least 8 consecutive residues selected from a sequence selected from the group consisting of TENFNMWKNNMVEQM, NFNMWKNNMVEQMQE, NMWKNNMVEQMQEDI, RKSIYIGPGRAFHTT, SIYIGPGRAFHTTGR, YIGPGRAFHTTGRII, GPGRAFHTTGRIIGD, GRAFHTTGRIIGDIR, GKNLPVLDQL, IAWYRMGDNCAIPITV, AGTYLRLV, LTSKAYQQG, CPIRTQPRWSYYDSF, INDWTEITQFILE, PRWPRWSYYDSFSAVSEDNLGFLMHAPAFETAGTYLRLVKINDWTEITQFILEHRARASCKYAL, ALYSLKIAGWHGPKP, and APAAPSNPG.

3. The T cell epitope peptide of claim 2, comprising twelve residues selected from a sequence selected from the group consisting of TENFNMWKNNMVEQM, NFNMWKNNMVEQMQE, NMWKNNMVEQMQEDI, RKSIYIGPGRAFHTT, SIYIGPGRAFHTTGR, YIGPGRAFHTTGRII, GPGRAFHTTGRIIGD, GRAFHTTGRIIGDIR, GKNLPVLDQL, IAWYRMGDNCAIPITV, AGTYLRLV, LTSKAYQQG, CPIRTQPRWSYYDSF, INDWTEITQFILE, PRWPRWSYYDSFSAVSEDNLGFLMHAPAFETAGTYLRLVKINDWTEITQFILEHRARASCKYAL, ALYSLKIAGWHGPKP, and APAAPSNPG.

4. The T cell epitope peptide of claim 2, further comprising a detectable label.

5. A method for detecting exposure of a mammal or bird to an antigen, which method comprises:

obtaining a biological sample from said subject, said sample containing T cells;

contacting said T cells with a T cell epitope peptide; and

detecting reaction between said T cell and said T cell epitope peptide.

6. The method of claim 5, wherein said T cell epitope peptide comprises a sequence of at least 8 consecutive residues selected from a sequence selected from the group consisting of TENFNMWKNNMVEQM, NFNMWKNNMVEQMQE, NMWKNNMVEQMQEDI, RKSIYIGPGRAFHTT, SIYIGPGRAFHTTGR, YIGPGRAFHTTGRII, GPGRAFHTTGRIIGD, GRAFHTTGRIIGDIR, GKNLPVLDQL, IAWYRMGDNCAIPITV, AGTYLRLV, LTSKAYQQG, CPIRTQPRWSYYDSF, INDWTEITQFILE, PRWPRWSYYDSFSAVSEDNLGFLMHAPAFETAGTYLRLVKINDWTEITQFILEHRARASCKYAL, ALYSLKIAGWHGPKP, APAAPSNPG, YSYFPSVI, IVPYIGPA, KQGYEGNFI, EQDPSGATTKSAMLTNLIIFGPGPVLNKNEV, and QEIYMQHTYPIS.

7. The method of claim 6, wherein said T cells are contacted with a plurality of T cell epitope peptides.

8. An assay kit for detecting exposure of a mammal or bird to an antigen, which kit comprises:

a T cell epitope peptide comprising a sequence of at least 8 consecutive residues selected from a sequence selected from the group consisting of TENFNMWKNNMVEQM, NFNMWKNNMVEQMQE, NMWKNNMVEQMQEDI, RKSIYIGPGRAFHTT, SIYIGPGRAFHTTGR, YIGPGRAFHTTGRII, GPGRAFHTTGRIIGD, GRAFHTTGRIIGDIR, GKNLPVLDQL, IAWYRMGDNCAIPITV, AGTYLRLV, LTSKAYQQG, CPIRTQPRWSYYDSF, INDWTEITQFILE, PRWPRWSYYDSFSAVSEDNLGFLMHAPAFETAGTYLRLVKINDWTEITQFILEHRARASCKYAL, ALYSLKIAGWHGPKP, APAAPSNPG, YSYFPSVI, KANSK, IDDFTNE, IVPYIGPA, KQGYEGNFI, EQDPSGATTKSAMLTNLIIFGPGPVLNKNEV, and QEIYMQHTYPIS; and

printed instructions for performing said assay.

9. The kit of claim 8, wherein said T cell epitope peptide further comprises a detectable label.

10. A vaccine composition for inducing an immune response in a bird or mammal, said composition comprising:

an effective amount of a B cell antigen;

an effective amount of a T cell epitope peptide comprising a sequence of at least 8 consecutive residues selected from a sequence selected from the group consisting of TENFNMWKNNMVEQM, NFNMWKNNMVEQMQE, NMWKNNMVEQMQEDI, RKSIYIGPGRAFHTT, SIYIGPGRAFHTTGR, YIGPGRAFHTTGRII, GPGRAFHTTGRIIGD, GRAFHTTGRIIGDIR, GKNLPVLDQL, IAWYRMGDNCAIPITV, AGTYLRLV, LTSKAYQQG, CPIRTQPRWSYYDSF, INDWTEITQFILE, PRWPRWSYYDSFSAVSEDNLGFLMHAPAFETAGTYLRLVKINDWTEITQFILEHRARASCKYAL, ALYSLKIAGWHGPKP, APAAPSNPG, YSYFPSVI, KANSK, IDDFTNE, IVPYIGPA, KQGYEGNFI, EQDPSGATTKSAMLTNLIIFGPGPVLNKNEV, and QEIYMQHTYPIS; and

a pharmaceutically acceptable carrier.

11. The vaccine composition of claim 10 wherein said B cell antigen comprises HSV gD2 or HIV_{SF2} gp120.

12. A method for increasing the number of T cells in a subject specific for a selected antigen, which method comprises:

obtaining a biological sample from said subject, said sample containing T cells;

contacting said T cells with a T cell epitope peptide;

culturing said T cells to specifically expand T cells reactive to said T cell epitope peptide; and

administering said T cells to said subject.

13. A method for determining T cell epitopes specific to an antigen, which method comprises:

preparing a plurality of peptide pools, wherein each pool comprises a plurality of peptides, wherein each peptide comprises eight amino acids, wherein the sequence of each peptide is selected from the sequence of the antigen;

obtaining a biological sample from a bird or mammal, said sample containing peripheral blood mononuclear cells;

contacting said peripheral blood mononuclear cells with said peptide pools;

culturing said peripheral blood mononuclear cells in the absence of xenogeneic serum; and

determining the mitogenic effect of said peptide pools.

14. The method of claim 13, wherein each peptide comprises twelve amino acids, and wherein each successive peptide overlaps the sequence of the preceding peptide by at least eight amino acids.

Figure 1

Met Lys Val Lys Gly Thr Arg Arg Asn Tyr
Gln His Leu Trp Arg Trp Gly Thr Leu Leu
Leu Gly Met Leu Met Ile Cys Ser Ala Thr
Glu Lys Leu Trp Val Thr Val Tyr Tyr Gly
Val Pro Val Trp Lys Glu Ala Thr Thr Thr
Leu Phe Cys Ala Ser Asp Ala Arg Ala Tyr
Asp Thr Glu Val His Asn Val Trp Ala Thr
His Ala Cys Val Pro Thr Asp Pro Asn Pro
Gln Glu Val Val Leu Gly Asn Val Thr Glu
Asn Phe Asn Met Trp Lys Asn Asn Met Val
Glu Gln Met Gln Glu Asp Ile Ile Ser Leu
Trp Asp Gln Ser Leu Lys Pro Cys Val Lys
Leu Thr Pro Leu Cys Val Thr Leu Asn Cys
Thr Asp Leu Gly Lys Ala Thr Asn Thr Asn
Ser Ser Asn Trp Lys Glu Glu Ile Lys Gly
Glu Ile Lys Asn Cys Ser Phe Asn Ile Thr
Thr Ser Ile Arg Asp Lys Ile Gln Lys Glu
Asn Ala Leu Phe Arg Asn Leu Asp Val Val
Pro Ile Asp Asn Ala Ser Thr Thr Thr Asn
Tyr Thr Asn Tyr Arg Leu Ile His Cys Asn
Arg Ser Val Ile Thr Gln Ala Cys Pro Lys
Val Ser Phe Glu Pro Ile Pro Ile His Tyr
Cys Thr Pro Ala Gly Phe Ala Ile Leu Lys
Cys Asn Asn Lys Thr Phe Asn Gly Lys Gly
Pro Cys Thr Asn Val Ser Thr Val Gln Cys
Thr His Gly Ile Arg Pro Ile Val Ser Thr
Gln Leu Leu Leu Asn Gly Ser Leu Ala Glu
Glu Glu Val Val Ile Arg Ser Asp Asn Phe
Thr Asn Asn Ala Lys Thr Ile Ile Val Gln
Leu Asn Glu Ser Val Ala Ile Asn Cys Thr
Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile
Tyr Ile Gly Pro Gly Arg Ala Phe His Thr
Thr Gly Arg Ile Ile Gly Asp Ile Arg Lys
Ala His Cys Asn Ile Ser Arg Ala Gln Trp
Asn Asn Thr Leu Glu Gln Ile Val Lys Lys
Leu Arg Glu Gln Phe Gly Asn Asn Lys Thr
Ile Val Phe Asn Gln Ser Ser Gly Gly Asp
Pro Glu Ile Val Met His Ser Phe Asn Cys
Arg Gly Glu Phe Phe Tyr Cys Asn Thr Thr
Gln Leu Phe Asn Asn Thr Trp Arg Leu Asn
His Thr Glu Gly Thr Lys Gly Asn Asp Thr
Ile Ile Leu Pro Cys Arg Ile Lys Gln Ile
Ile Asn Met Trp Gln Glu Val Gly Lys Ala
Met Tyr Ala Pro Pro Ile Gly Gly Gln Ile

Figure 1 (continued)

```
Ser Cys Ser Ser Asn Ile Thr Gly Leu Leu
Leu Thr Arg Asp Gly Gly Thr Asn Val Thr
Asn Asp Thr Glu Val Phe Arg Pro Gly Gly
Gly Asp Met Arg Asp Asn Trp Arg Ser Glu
Leu Tyr Lys Tyr Lys Val Ile Lys Ile Glu
Pro Leu Gly Ile Ala Pro Thr Lys Ala Lys
Arg Arg Val Val Gln Arg Glu Lys Arg
```

INTERNATIONAL SEARCH REPORT

Interna l Application No
PCT/US 93/11703

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/00 C07K7/08 C07K7/06 A61K38/04 A61K39/00 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. ACQUIRED IMMUNE DEFIC. SYNDR., vol.2, no.1, 1989 pages 21 - 27 MODROW, S. ET AL. 'Use of synthetic oligopeptides in identification and characterization of immunological functions in the amino acid sequence of the envelope protein of HIV-1' * whole disclosure, esp. tables 1 and 2, peptide 100-112 * ---	1-9
X	EP,A,0 429 816 (F. HOFFMANN-LA ROCHE) 5 June 1991 * claims 1 and 2 * ---	10,11
A	WO,A,89 02277 (UNIV. OF TEXAS) 23 March 1989 * claims * --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
15 September 1994	01 -02- 1995
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer HERMANN R.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interna l Application No
PCT/US 93/11703

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,4 943 628 (ROSEN, J.I. & WARNER, J.F.) 24 July 1990 * whole disclosure * ---	1-12
A	WO,A,92 21377 (SYNTELLO VACCIN DEVELOPMENT AB) 10 December 1992 * whole disclosure * -----	1-12

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int ..tional application No.
PCT/US93/11703

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims 1 - 12(all partially)
2. Claims 1 - 12 " "
3. Claims 1 - 12 " "
4. Claims 1 - 12 " "
5. Claims 1 - 12 " " See annex.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-12 (all partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US93/11703

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

LACK OF UNITY OF INVENTION

1. Claims: 1-12 (all partially)	T-cell epitopes derived from HIV gp120, containing the sequence NMWKNNMVEQM (the first three peptides of claim 1), and subject-matter relating to such epitopes.
2. Claims: 1-12 (all partially)	T-cell epitopes derived from HIV gp120, containing the sequence GRAFHTT (peptides four to eight of claim 1), and subject-matter relating to such epitopes.
3. Claims: 1-12 (all partially)	T-cell epitopes derived from HSV (peptides 9 to 17 of claim 1), and subject-matter relating to such epitopes. (Please note that these peptides do not share common structural features; further non-unity objections may arise if the common concept (HSV T-cell epitopes) turns out to be known).
4. Claims: 1-12 (all partially)	T-cell epitopes derived from tetanus toxin (peptides 17 to 24 of claim 1), and subject-matter relating to such epitopes. (Please note that these peptides do not share common structural features; further non-unity objections may arise if the common concept (TT T-cell epitopes) turns out to be known).
5. Claims: 13-14	A method for determining T-cell epitopes.

INTERNATIONAL SEARCH REPORT

Interna l Application No
PCT/US 93/11703

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0429816	05-06-91	AU-B-	637841	10-06-93
		AU-A-	6557190	09-05-91
		JP-A-	3173830	29-07-91
WO-A-8902277	23-03-89	AU-A-	2914889	17-04-89
		US-A-	5128319	07-07-92
US-A-4943628	24-07-90	NONE		
WO-A-9221377	10-12-92	AU-A-	1906592	08-01-93
		AU-B-	7169494	24-11-94
		EP-A-	0594638	04-05-94
		JP-T-	6510025	10-11-94
		US-A-	5346989	13-09-94